ACTIVATION OF MURINE LYMPHOCYTES BY 2-MERCAPTOETHANOL AND RELATED THIOL COMPOUNDS AND ITS MECHANISM. II. EFFECT OF THIOL COMPOUNDS ON THE VIABILITY OF THE LYMPHOCYTES UNDER A CYSTINE-DEFICIENT CULTURE CONDITION

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When murine lymphocytes were cultured in a cystine-deficient RPMI-1640 medium containing 10% fetal calf serum, approximately 70% of the cells died in 48 h. In the presence of 5×10^{-4} M L-cystine, 70–80% of the lymphocytes remained viable under the same condition. The decrease of the viability was also effectively inhibited when cystine was replaced by the following thiol compounds; 2-mercaptoethanol (2-ME), dithiothreitol (DTT), cysteamine and glutathione (GSH). Oxidized DTT, an intramolecular disulfide that was resistant to the reduction by the lymphocytes, did not show a protective effect in contrast to DTT. On the other hand, 2-hydroxyethyl disulfide was readily reduced to 2-ME by the lymphocytes and improved lymphocyte viability as effectively as 2-ME. These observations suggest that thiol groups are responsible for the improvement of lymphocyte viability under a cystine-deficient condition. The viabilities of both T cells and B cells were equally improved by 2-ME. The intracellular level of GSH remained constant in the presence of cystine but dropped rapidly in its absence. 2-ME could not reverse this decrease of GSH level. These protective effects of thiol compounds on lymphocyte viability are discussed in relation to their capacities to activate murine lymphocytes.

Keywords — thiol compound; cystine-deficiency; murine lymphocyte; cell viability; glutathione; lymphocyte activation

INTRODUCTION

It has been demonstrated that 2-mercaptoethanol (2-ME) and other thiol compounds markedly augment various *in vitro* lymphocyte reactions like antibody responses,^{1–3)} proliferative responses to mitogens^{3,4)} and the induction of cytotoxic T cells.^{5,6)} We have observed that a correlation exists between the augmenting effects of various thiol compounds on the antibody response and those on the mitogenic responses.³⁾ We have recently reported that one of the major roles of 2-ME in the augmentation of the antibody response is to stimulate the transport of cystine into murine lymphocytes.^{7–9)} Cystine was shown to be essential for the proliferation and differentiation of murine lymphocytes and is contained in RPMI-1640 medium at a suboptimal level (0.19 mM), while more than 1 mM cystine is necessary for optimal antibody response.⁷⁾ During the course of these investigations, we observed that the viability of the lymphocytes was significantly improved in the pre-

sence of various thiol compounds, particularly under a cystine-deficient condition. Some authors have reported that cultured human fibroblasts rapidly died when they were transferred to a cystine-deficient conditions.¹⁰⁾ Various antioxidants including thiol compounds were shown to have protective effects in these systems.^{10,11)} In the present paper, we investigated the effects of various thiol compounds on the viability of murine lymphocytes cultured in a cystine-deficient condition to elucidate one of the mechanisms of lymphocyte activation by these compounds.

MATERIALS AND METHODS

Animals — Female BALB/c mice (6 weeks of age) were purchased from Shizuoka Agricultural Corporation of Experimental Animals (Shizuoka) and were used during 10–15 weeks of age.

Materials — Materials used in this work were obtained from the following sources: 2-ME and L-cystine dihydrochloride (Tokyo Kasei);

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sodium thioglycollate (Ishizu Seiyaku); dithiothreitol (DTT), oxidized DTT (DTT_{ox}), cysteamine and glutathione (GSH, Sigma); glutathione reductase (Boehringer Mannheim); fetal calf serum (FCS, GIBCO); RPMI-1640 medium (Nissui Seiyaku). 2-Hydroxyethyl disulfide (2-ME_{ox}) was a gift from Dr. T. Iso (Santen Seiyaku). RPMI-1640 medium devoid of cystine and GSH was prepared in our laboratory according to the reported composition.¹²⁾ The cystine- and GSH-free culture medium containing 10% FCS was referred to cystine-deficient culture medium. Cyst(e)ine content of this medium was estimated to be less than 2×10^{-6} M. The culture medium was usually supplemented with penicillin G (50 units/ml) and streptomycin (50 μ g/ml).

Lymphocyte Culture — Erythrocytes (RBC)-depleted spleen cells were prepared by treatment with Tris-NH₄Cl.¹³⁾ RBC-depleted spleen cells of BALB/c mice (6×10^6) were cultured at 37 °C under 5% CO₂ in air in 1 ml of cystine-deficient RPMI-1640 medium containing 10% FCS for 48 h in the presence or absence of the indicated agents. Viable cells were enumerated by the trypan blue exclusion test. Total number of cells did not change significantly during the culture period. Data were presented as the mean values of duplicate experiments. Observed values did not deviate over 10% of the mean values.

Preparation of T Cell- or B Cell-Enriched Spleen Lymphocytes — T cell- or B cell-enrichment was performed by treating spleen cells with anti-mouse Ig or anti-Thy-1,2 (Olac 1978), respectively in the presence of guinea pig complement as reported previously.⁷⁾

Determination of Intracellular GSH — RBC-depleted spleen lymphocytes (8×10^6) were cultured in the presence or absence of 5×10^{-4} M cystine. At the indicated time, GSH was extracted from the harvested cells with 10% trichloroacetic acid and determined enzymatically using glutathione reductase as reported by Brehe and Burch.¹⁴⁾

RESULTS

Rapid Decrease of the Lymphocyte Viability in the Cystine-Deficient Condition

As shown in Fig. 1, the viability of RBC-depleted spleen cells gradually decreased to 70–80% in 48 h when the cells were cultured in RPMI-1640 medium containing 10% FCS.

RPMI-1640 medium contained 65 μ g/ml cystine dihydrochloride and 1 μ g/ml GSH. However, the cells were found to die much more rapidly when either cystine or cystine plus GSH

FIG. 1. *Effect of Depletion of Cystine or GSH on the Viability of Murine Lymphocytes*

RBC-depleted murine spleen cells were cultured in RPMI-1640 medium containing 10% FCS. Where indicated, cystine (65 μ g/ml) or GSH (1 μ g/ml) was omitted from the culture medium. ○, complete medium; ●, without cystine; □, GSH; △, without both cystine and GSH.

FIG. 2. *Effect of Cystine Concentrations on the Viability of Murine Lymphocytes*

RBC-depleted murine spleen cells were cultured in cystine-deficient RPMI-1640 medium containing 10% FCS in the presence of varying concentrations of cystine for 48 h.

was omitted from the culture medium. Removal of GSH alone did not significantly affect the change of the lymphocyte viability. RBC were removed from murine spleen cells in order to avoid the contamination of GSH and other thiols contained in the cells. In the following experiments, RPMI-1640 medium that was devoid of both cystine and GSH was used as the cystine-deficient medium. When increasing concentrations of cystine were added to the cystine-deficient medium containing 10% FCS, the cell viability after 48 h gradually improved and reached a plateau at 5×10^{-4} M cystine as shown in Fig. 2.

Effect of 2-ME on the Viability of Murine Lymphocytes Cultured under the Cystine-Deficient Condition

As illustrated in Fig. 3, 2-ME showed a protective effect against the decrease of the viability of murine lymphocytes that were cultured for 48 h in the cystine-deficient medium. 2-ME was most effective at 10^{-5} M, which was also optimal for the augmentation of the antibody response.³⁾ The protective effect was similarly observed in both serum-containing and serum-free culture medium, although the cells died more rapidly in the absence of FCS than in its presence. These ob-

FIG. 3. *Effect of 2-ME on the Viability of Murine Lymphocytes Cultured in Cystine-Deficient Medium in the Presence or Absence of FCS*

RBC-depleted murine spleen cells were cultured in cystine-deficient RPMI-1640 medium containing no or 10% FCS in the presence of varying concentrations of 2-ME for (○) 24 h (●) 48 h, (△) 72 h or (□) 96 h.

servations suggest that the protective effect of 2-ME was enhanced by some components contained in the serum. The following experiments were performed by using the cystine-deficient medium containing 10% FCS because the protective effect of thiol compounds could be observed more clearly.

Effect of Various Thiol and Disulfide Compounds on Lymphocyte Viability under the Cystine-Deficient Condition

Various thiol compounds were examined for their protective effects on the viability of murine lymphocytes that were cultured in the cystine-deficient medium. As shown in Fig. 4, cell viability improved with the indicated order of use of 2-ME, DTT and cysteamine. Thioglycollate failed to show the protective effect. These protective effects of thiol compounds correlated well with their stimulating effects on the proliferative response and the antibody response.³⁾ GSH at 10^{-3} M showed a comparable effect of 10^{-5} M 2-ME or 5×10^{-4} M cystine as shown in Fig. 4, although it could not stimulate the antibody response when added to RPMI-1640 medium containing 10% FCS.³⁾ This discrepancy will be discussed later.

FIG. 4. *Effect of Various thiol Compounds on the Viability of Murine Lymphocytes Cultured in Cystine-Deficient Medium*

RBC-depleted spleen cells were cultured in cystine-deficient RPMI-1640 medium containing 10% FCS for 48 h in the presence of the indicated thiol compounds.

FIG. 5. Comparison between Thiol and Disulfide Compounds for Their Capacities to Improve the Lymphocyte Viability under the Cystine Deficient Conditions

RBC-depleted spleen cells were cultured for 48 h in cystine-deficient medium containing 10% FCS in the presence of the indicated thiol or disulfide compounds.

Fig. 5 shows the effect of disulfide compounds on lymphocyte viability under the cystine-deficient condition in comparison with those of their thiol forms. 2-ME_{ox} improved the cell viability as effectively as its reduced form, 2-ME. But DTT_{ox}, an intramolecular disulfide form of DTT was completely ineffective in contrast to DTT. In the previous experiments, we confirmed that 2-ME_{ox} was readily reduced to 2-ME in the lymphocyte culture.⁹⁾ But DTT_{ox} was resistant to the reduction by the lymphocytes and was assumed to behave as the disulfide form in the lymphocyte culture.¹⁵⁾ These results imply that thiol groups are responsible for the improvement of the lymphocyte viability under the cystine-deficient condition.

Because thiol compounds possess activities as antioxidants, it was then investigated whether the effect of thiols can be replaced by other antioxidants or scavengers of active oxygen and its radicals. We tested the following agents; superoxide dismutase, catalase, butylated hydroxytoluene, *N,N'*-diphenyl-1,4-phenylenediamine, vitamin E and levamisole. All these agents, however, failed to improve the lymphocyte viability in contrast to thiol compounds (data not shown).

Protective Effect of 2-ME on the Viability of T Cells or B Cells Cultured under the Cystine-Deficient Condition

T cell-enriched or B cell-enriched murine

FIG. 6. Effect of 2-ME on the Viability of T Cells or B Cells Cultured under the Cystine-Deficient Conditions

T cells or B cells were enriched from RBC-depleted murine spleen cells as described under Materials and Methods. These cells (6×10^6) were cultured in the cystine-deficient medium containing 10% FCS for 48 h in the presence or absence of 5×10^{-4} M cystine (Cys) or 10^{-5} M 2-ME.

FIG. 7. Change of GSH Levels in Murine Lymphocytes Cultured in the Presence or Absence of Cystine

RBC-depleted murine spleen cells (8×10^6) were cultured in the cystine-deficient medium containing 10% FCS. Where indicated, (○) none, (▲) 2-ME (10^{-5} M), or (●) cystine (5×10^{-4} M) was added to the culture medium. GSH contents were assayed enzymatically (see Materials and Methods).

lymphocytes were cultured under the cystine-deficient condition in order to investigate which subpopulation is more susceptible to cystine-deficiency. As shown in Fig. 6, the viability of B cells decreased more markedly than T cells. The viability of both subpopulations, however, were improved to the same extent by 2-ME.

Effect of 2-ME on the Level of GSH in Murine Lymphocytes Cultured under the Cystine-Deficient Condition

Cystine that was transported into the cells is thought to be converted, in part, into GSH that functions as a protective agent against oxidative damages of the cells. We confirmed that more than 50% of transported cystine was converted to GSH in 1 h in murine lymphocytes.⁸⁾ As shown in Fig. 7, the intracellular GSH level remained constant for 48 h in the presence of 5×10^{-4} M cystine, but dropped rapidly within 8 h when the lymphocytes were transferred to the cystine-deficient medium. This rapid decrease of GSH was not reversed by the addition of 2-ME to the cystine-deficient medium, indicating that the 2-ME-effect is not due to the maintenance of GSH level in the cells.

DISCUSSION

In the present report, we demonstrated that various thiol compounds markedly improved the viability of murine lymphocytes cultured under the cystine-deficient condition. We have reported that antibody response and proliferative response in murine lymphocytes were markedly augmented by thiol compounds with the following order of effectiveness; $2\text{-ME} \geq \text{DTT} > \text{cysteamine}$.³⁾ GSH and thioglycollate were almost ineffective. The same order of activities was observed in the improvement of cell viability under the cystine-deficient condition except for GSH, which was shown to be as effective as 2-ME in this case. According to our unpublished observation, GSH at 1 mM could elicit antibody response *in vitro* as effectively as 1 mM cystine when added to the cystine-deficient medium. Therefore, GSH could be used as a cysteine source in this case. The effect of GSH, however, was abrogated in the presence of cystine (for example, in RPMI-1640 medium). This can be explained in the following way. In the presence of cystine, we observed that GSH was much more rapidly converted to oxidized GSH (GSSG) than in its absence. GSSG was reported

to be cytotoxic and was not utilized by murine lymphocytes in the antibody response *in vitro* (ref. 16 and our unpublished data). Under the cystine-deficient condition, GSH is thought to exist as the reduced form for a longer period and would be used for maintaining cell viability. The improvement of lymphocyte viability by thiol compounds would not be due to the mitogenic effects of these compounds because no significant deoxyribonucleic acid (DNA) synthesis was elicited under the cystine-deficient condition.⁷⁾

It was found that the intracellular GSH level was retained in the presence of cystine but decreased rapidly under the cystine-deficient condition. We have observed that cyst(e)ine was efficiently converted to GSH after it was taken up by the lymphocytes.⁸⁾ It has been known that intracellular GSH plays an important role in protecting the cells against oxidative damages as a substrate of GSH peroxidase.¹⁷⁾ Hoffeld has reported that *in vitro* antibody responses are augmented by agents that inhibit lipid peroxidation.¹⁸⁾ 2-ME could not reverse the decrease of GSH level under cystine-deficiency (Fig. 7). Therefore, it is likely that some roles of GSH can be replaced by 2-ME and other thiols in the lymphocytes. It has been reported that various thiol compounds including 2-ME and cysteamine could be substrates of GSH peroxidase with low efficiency.¹⁹⁾ When human fibroblasts were cultured under the cystine-deficient condition, their viability was markedly improved by various antioxidants, including thiol compounds.^{10,11)} In murine lymphocytes, however, only thiol compounds were effective and other non-thiol antioxidants had no effects. Although the role of thiol compounds as antioxidants can not be ruled out, these compounds might act on the cells by some mechanisms other than protecting the cells against oxidative damages. Besides the role as an antioxidant, GSH would be a key metabolite in various biological systems. For instance, it has been proposed by Oliver *et al.*²⁰⁾ that GSH plays an important role in regulating the assembly and disassembly of microtubules by binding to some regulatory protein in polymorphonuclear leukocytes. Various thiol compounds might be able to act as a substitute for GSH in such systems. The above results suggest that intracellular thiol contents must be maintained at an appropriate level for lym-

phocytes to be activated. Thiol compounds would contribute to the maintenance of the thiol level in the cells by stimulating cystine uptake⁷⁾ or by substituting the role of endogenous thiols such as GSH and cysteine.

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